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TITLE: Role of Hypomethylating Agents in the Treatment of Bone Marrow Failure

PRINCIPAL INVESTIGATOR: Benjamin L. Ebert, MD, PhD

CONTRACTING ORGANIZATION: The Brigham and Women's Hospital, Inc
Boston, MA 02115

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Benjamin L. Ebert, MD, PhD

E-Mail:bebert@partners.org

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The Brigham and Women's Hospital,
Inc.
75 Francis Street
Boston, MA 02115-6110

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

Hypomethylating agents, 5-azacytidine and decitabine, are effective therapies for the treatment of myelodysplastic syndromes (MDS), but the genetic basis of response to these agents is unknown. We hypothesize that, given the heterogeneous clinical response to hypomethylating agents in MDS patients and the diversity of molecular lesions that drive the clinical phenotype of MDS, specific mutations alter response to therapy. The identification of mutations that predict response to therapy could have an immediate clinical impact, enabling physicians to select the patients most likely to respond, to determine the duration of treatment, and to monitor disease progression. Such therapy has the potential to alter the course of disease for patients with bone marrow failure and clonal somatic mutations that occur in myeloid malignancies.

15. SUBJECT TERMS
Myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), hypomethylating agents (HMA), azacitidine, decitabine, TET2 mutations

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ANNUAL TECHNICAL REPORT

Ebert, Benjamin L.

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

Hypomethylating agents, 5-azacytidine and decitabine, are effective therapies for the treatment of myelodysplastic syndromes (MDS), but the genetic basis of response to these agents is unknown. We hypothesize that, given the heterogeneous clinical response to hypomethylating agents in MDS patients and the diversity of molecular lesions that drive the clinical phenotype of MDS, specific mutations alter response to therapy. The identification of mutations that predict response to therapy could have an immediate clinical impact, enabling physicians to select the patients most likely to respond, to determine the duration of treatment, and to monitor disease progression. Such therapy has the potential to alter the course of disease for patients with bone marrow failure and clonal somatic mutations that occur in myeloid malignancies.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), hypomethylating agents (HMA), azacitidine, decitabine, TET2 mutations

3. ACCOMPLISHMENTS:

What were the major goals of the project?

In Specific Aim 1, we propose to identify mutations in MDS that mediate sensitivity and resistance to hypomethylating agents. In Aim 1, we proposed to examine the full spectrum of known MDS mutations in pre-treatment samples from MDS patients who received hypomethylating agents. The approach was to examine somatic mutations using hybrid capture followed by Illumina sequencing. Our goal was to identify mutations that can predict sensitivity or resistance to hypomethylating agents. In Aim 1B, genetic studies will be complemented with whole-genome analysis of methylation and hydroxymethylation status.

Aim 1A: Identify mutations in MDS that mediate sensitivity and resistance to hypomethylating agents

> We completed the goal of this aim prior to initiation of funding. Progress was more rapid than expected and was under competition, so the proposed experiments were completed before funding for the project actually began. The results have been published, Bejar et al., Blood 2014.

Aim 1B: Perform analysis of methylation and hydroxymethylation in MDS samples

> This has been the major focus of our work over the past year. We have completed reduced representation bisulfite sequencing of 66 MDS cases to analyze DNA methylation. We completed RNA Sequencing of the samples as well. Data analysis of the methylation is complete. Analysis of hydroxymethylation is ongoing.

In Specific Aim 2, we propose an in vivo RNA interference screen to identify mutations that sensitize cells to hypomethylating agents. Using RNA interference screening in primary human bone marrow progenitor with assays of hematopoietic differentiation, we identified *RPS14* as a critical gene for the phenotype of the 5q-syndrome. More recently, we have developed approaches for pooled *in vitro* and *in vivo* RNA interference screens to detect genes that influence clonal dominance. In Specific Aim 2, we will employ these approaches to

find mutations and therapeutic targets that alter sensitivity to hypomethylating agents. We will perform pooled, in vivo shRNA screens, in the presence or absence of acacitidine. We will seek to identify and validate genes and therapeutic targets that later sensitivity to azacitidine in order to identify novel combination therapies that could have increased efficacy in MDS patients.

Aim 2A: Using an *in vivo* shRNA screening approach, target genes with loss of function mutations and genes encoding therapeutic targets.

We have focused on generating the models for in vivo screens and developing shRNA and CRISPR libraries to execute the screens.

Aim 2B: Validate hits from the functional screen.

Pending results of the screen.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

We have made major progress in completion of Aim 1. As noted above, Aim 1A was largely completed earlier than expected. We utilized targeted capture and highly parallel quantitative sequencing to examine samples from MDS and CMML patients collected prior to treatment with an azacitidine or decitabine containing regimen. Mutations were examined for association with response to treatment and overall survival. The overall response rate was 47% in this cohort, with no difference observed between treatment groups (p=0.96). *TET2* mutations predicted response (odds ratio of response [OR] 1.99, p = 0.036 [95% CI, 1.05-3.80], p = 0.036; adjusted OR [adjOR] 1.98 [1.02-3.85], p=0.044) when mutations unlikely to be detected by Sanger sequencing (allele fraction of < 10%) were treated as wildtype. The response rate was highest in the *ASXL1* wildtype subset of *TET2* mutant patients (OR 3.65, p = 0.009 , adjOR 3.64 [1.35-9.79], p=0.011). Analysis of overall survival in 146 patients from cohorts with survival data collected identified mutations in *TP53* (n = 31; hazard ratio [HR] 2.01 [95% CI, 1.29-3.14], p = 0.002; adjusted HR [adjHR] 1.91 [1.20-3.05], p=0.007) and *PTPN11* (n = 6; HR 3.26 [1.41-7.58], p = 0.006, adjHR 2.47 [0.98-6.26], p=0.056) as adverse. Patients with complex karyotypes had a high rate of *TP53* mutation (63%) which was associated with shorter overall survival compared to *TP53* unmutated complex karyotype patients (median 0.9 years vs. 1.3 years, p=0.030). To test the hypothesis that *TET2* loss of function could result in sensitivity to hypomethylating agents, we performed murine competitive bone marrow transplant experiments followed by treatment with azacitidine.

Aim 1B: Analysis of DNA methylation and hydroxymethylation was the primary focus of this first year of funding. The findings form Aim 1A demonstrated that *TET2* mutant MDS cases are sensitive to therapy with hypomethylating agents. We therefore sought to determine the methylation consequences of *TET2* mutations.

We sought to characterize the effects of somatic *TET2* mutation on DNA methylation in a well-annotated set of samples from a single disease, MDS, by reduced-representation bisulfite sequencing (RRBS). We analyzed genomic DNA isolated from total bone marrow mononuclear cells from 66 MDS cases matched for *TET2* mutation status, somatic mutations in other genes, Analysis of all well-covered regions showed that, as expected, MDS samples were highly methylated regardless of genotype, but we observed a small, but significant, shift towards increased methylation (≥10% change across 1kb tiles) in the *TET2*-mutant samples versus WT.

We next sought to determine the specific genomic regions most affected by loss of *TET2*. To control for differences in region size, we normalized differential methylation in each region to CpG content of that region (Figure 1). While the majority of total CpGs were located in promoters, CpG islands, and enhancers, these regions contained only a small fraction of differentially methylated CpGs, and differential methylation in these regions was evenly split between the *TET2*-mutant and *TET2*-WT samples. Although promoters are enriched for hmC and Tet1 binding in murine ES cells, we found no significant difference in methylation between groups at promoters, which had low levels of methylation overall (Figure 1c, d). Methylation at enhancers has been implicated in regulation of transcriptional control[5], but we saw no significant differences between *TET2* mutant and WT groups in the overlap of our methylation data and two separate enhancer datasets. In contrast, non-promoter intron-exon boundaries showed marked enrichment for differential methylation despite containing a relatively low fraction of total CpGs (Figure 1e, f).

The finding that intron-exon boundaries contained the highest proportion of relative differential methylation in our samples is consistent with reports that 5-hmC is enriched at intron-exon boundaries in neuronal tissues and is depleted in gene bodies with loss of *Tet2* in murine ES cells. We examined the precise location of differential methylation relative to the intron-exon boundary by plotting the relative methylation level of well-covered differentially methylated CpGs within 100bp of an intron-exon boundary relative to distance the intron exon boundary. We saw no bias in differential methylation towards 5' versus 3' boundaries, but observed a consistent gain in methylation in the *TET2* mutant group across this region (Figure 1g).

We next sought to investigate whether sites of altered methylation occur at hydroxymethylated loci. We generated isogenic TF-1 cell line clones using CRISPR-Cas9 with and without targeted, homozygous disruption of the *TET2* gene. The two clones generated with *TET2* inactivation had reduced TET2 function as assessed by overall hmC content. To ensure that methylation changes were consistent between MDS samples and the TF1 lines, we validated a set of highly differentially methylated DMRs from MDS samples in TF1 lines by methyl-DNA-immunoprecipitation (MeDIP) followed by qPCR. To identify specific sites of altered hmC content, we performed hmC sequencing on genomic DNA using a reduced-representation hydroxymethylation profiling approach (RRHP, Zymo). Consistent with an overall loss of hmC in *TET2*-mutated cells, we observed a ten-fold decrease in the number of reads and unique sites captured in the *TET2*-mutant cells compared to *TET2*-WT. Interestingly, of the sites captured with significant coverage in the *TET2*-mutant samples (n=48), approximately half (n=22) mapped to the mitochondrial chromosome, which contains both mC and hmC.

To understand the relationship between 5-hmC and methylation in TET2 mutant cells, we examined the overlap of RRHP and WGBS sequencing data. The majority of overlapping sites occurred in regions identified as non-differentially methylated between *TET2*-mutant and -WT samples by bisulfite sequencing (Supplemental Figure 6), which may reflect the inability of WGBS to discriminate between hmC and mC. We then focused on intron-exon regions within the overlapping dataset, and observed a significant increase in hmC in *TET2*-WT cells at intron-exon regions that were also hypermethylated in *TET2*-mutant samples (Figure 2b). While the influence of hmC on methylation was modest, though highly significant, the hmC mark may have a short half-life relative to DNA methylation. Within individual genes, regions highly differentially methylated between *TET2* wildtype and *TET2*-mutant groups by WGBS correspond to hmC peaks in *TET2* wildtype cells, suggesting that increased methylation in *TET2* mutant samples is directly due to loss of TET2 function (Figure 2c).

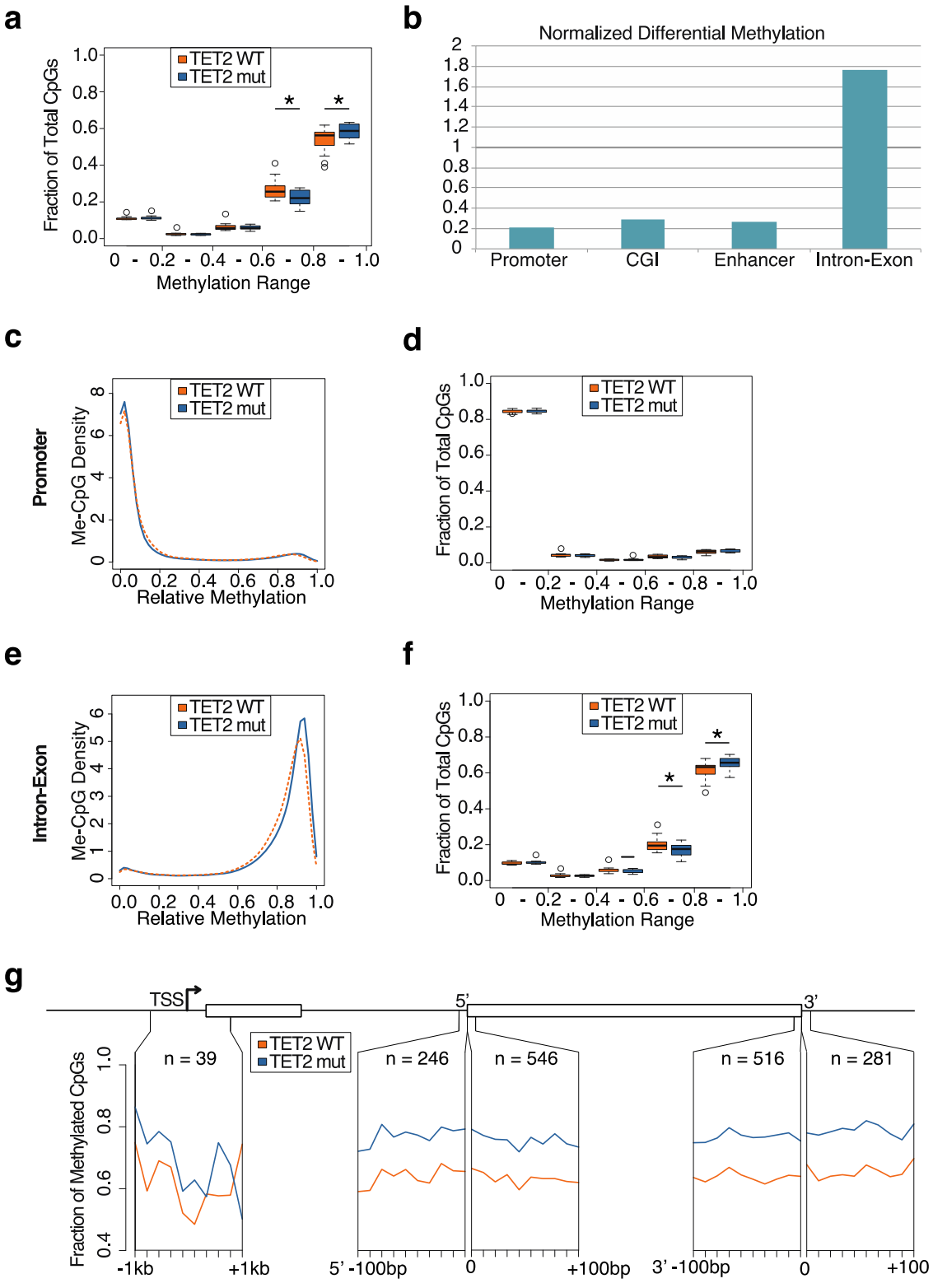

Figure 1: Overall and intragenic hypermethylation in *TET2*-mutant samples. Overall and intragenic hypermethylation in *TET2*-mutant samples. Well-covered CpGs were binned according to level of relative methylation and statistics were performed between TET2 mutant and TET2 WT groups for each bin (e; paired t-test, * indicates $p<0.05$) (a); Differential methylation was normalized to region size for the regions indicated, and was calculated as the fraction of differentially methylated CpGs in a given region relative to the fraction of total CpGs in that region (b); Promoter methylation (c,d) and intron-exon methylation (e, f) shown as comparison of

average methylation between TET2 mutant and TET2 WT groups (c, e) and relative methylation of each group as a function of density (d, f); Differential methylation across the promoter region and the intron-exon region was averaged and plotted as a function of distance from the TSS and the 5' and 3' exon boundary, respectively.

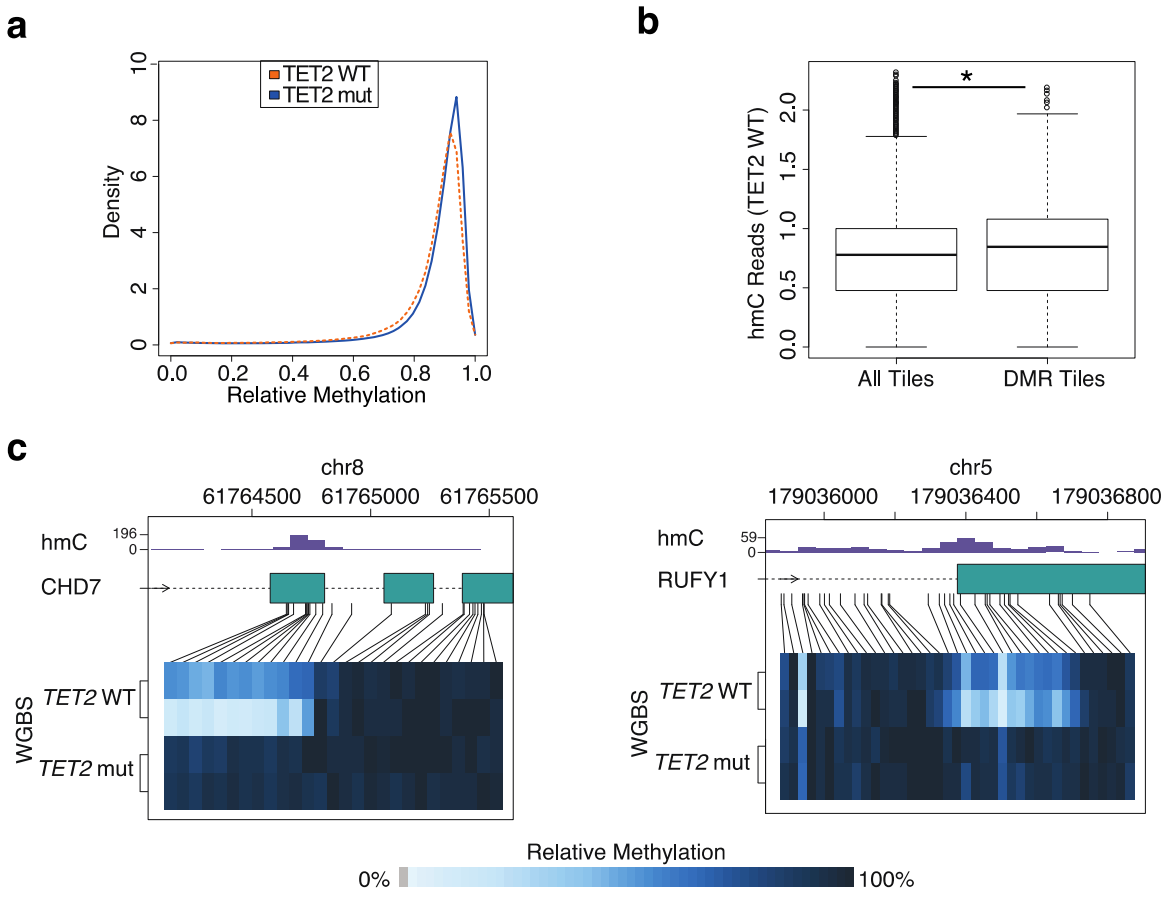

Figure 2: Sites of intragenic hypermethylation in *TET2*-mutant MDS samples are enriched for hydroxymethylcytosine in *TET2*-WT samples. Comparison of intron-exon WGBS results between *TET2*-wildtype and *TET2*-mutant groups as a function of density (a). hmC reads by RRHS in *TET2*-wildtype TF1 cells were compared to hypermethylated intron-exon regions ("DMR tiles") and all intron exon regions ("All Tiles") (b; two sample t-test, *** indicates $p \leq 0.0001$). WGBS methylation data and RRHS hmC data (in *TET2*-wildtype) are shown for selected regions (c).

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

A major focus of our work in the next year will be to complete our analysis of methylation and hydroxymethylation. The analysis described above demonstrates that *TET2* mutations result in altered DNA methylation and hydroxymethylation at intragenic sites near exon-intron junctions. A goal of the coming year will be to perform RNA sequencing to determine whether altered methylation at intron-exon junctions results in

differential splicing. This will be followed by validation studies to validate specific sites that have differential methylation, specific sites that have differential hydroxymethylation, and specific sites with alternative splicing.

In parallel, we are working on the pooled genetic screens described in Aim 2. The approach is complementary but independent of the studies described in Aim 1. Our goal will be to complete a screen and have an initial hit for validation by the end of the second year.

4. IMPACT: This component is used to describe ways in which the work, findings, and specific products of the project have had an impact during this reporting period. Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

The results of our studies have the potential to influence clinical practice. Patients with mutations that have high sensitivity to hypomethylating agents may be prioritized for treatment with these agents. This has the potential to increase the overall therapeutic efficacy of hypomethylating agents in clinical practice. Specifically, we have found that *TET2* mutations sensitize cells to hypomethylating agents based on human genetics studies, and that *TET2* mutations cause increased methylation at intragenic regions that may cause altered mRNA splicing. These areas of hypermethylation would be expected to be demethylated following exposure to azacitidine or decitabine, resulting in reversal of the oncogenic effects of *TET2* mutation. This informs biology of *TET2* mutations, the mechanism of action of hypomethylating agents, and the identification of myelodysplastic syndrome patients most likely to respond to these drugs.

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Nothing to report

6. PRODUCTS: List any products resulting from the project during the reporting period. Examples of products include:

- **Publications, conference papers, and presentations**

Publication based on work described in Aim 1A and that was completed prior to initiation of funding: Bejar R, Lord A, Stevenson K, Bar-Natan M, Pérez-Ladaga A, Zaneveld J, Wang H, Caughey B, Stojanov P, Getz G,

Garcia-Manero G, Kantarjian H, Chen R, Stone RM, Neuberg D, Steensma DP, **Ebert BL**. TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients. Blood. 2014 Oct 23;124(17):2705-12.

- **Website(s) or other Internet site(s)**

Nothing to report

- **Technologies or techniques**

Nothing to report

- **Inventions, patent applications, and/or licenses**

Provisional patent application: international publication number WO 2014/183122 A1. Methods for determining response to a hypomethylating agent.

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Benjamin L. Ebert, MD, PhD
Project Role:	Principle Investigator
Nearest person month worked:	0.6 CM
Contribution to Project:	Oversight of all aspects of the project

Name:	Andrew Guirguis
Project Role:	Postdoctoral Fellow
Nearest person month worked:	5CM
Contribution to Project:	Dr. Guirguis has is leading all of the laboratory-based efforts for this project.
Funding Support:	The Haematology Society of Australia and New Zealand

Name:	Michelle Chen
Project Role:	Research Technician
Nearest person month worked:	5 CM
Contribution to Project:	Ms. Chen has assisted with functional studies described in Aim 2

Name:	Alexander Silver
Project Role:	Research Technician

Nearest person month worked: 3 CM

Contribution to Project: Mr. Silver has assisted with functional studies described in Aim 2

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

EBERT, BENJAMIN - OTHER SUPPORT

Ended Projects since award activation

Novel Therapeutic Strategies in Human Myeloid Leukemias—Project 2
P01 CA066996 (Griffin) 4/18/09-3/31/14 1.44 cal.
DFCI NIH/NCI $210,337
Amey Ehrenzweig
Dana Farber Cancer Institute
450 Brookline Avenue
Boston, MA, 02215
The aims of this project are 1) Generation and characterization of accurate models of leukemia mediated by mutated FLT3 using knock-in strategies. 2) Characterize the cooperative effects of these accurate genotypic models of FLT3-ITD mediated disease with crosses to other germline alleles. 3) Develop accurate murine models of JAK2V617F mediated MPD.
THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

Artificial, Humanized Stem Cell Niches
R01EB012521-01A1 (Parkekkadan) 08/01/11-06/30/15 0.60 cal.
Massachusetts General Hospital; NIH $56,055
Thekla Diehl/Senior Grant Administrator
55 Fruit Street
Boston MA 02114
The goal of this project is to examine the in vivo potential of hematopoietic stem cells cultured in 3-dimensional, bioengineered, co-culute bone marrow niches. We expect these studies to create a platform for examination ofinteractions between the hematopoietic cells and bone marrow stromal cells.
THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

Identification of genetic dependencies in leukemia stem cells
064 - Medical Research Award (Ebert) 01/01/12-12/31/14 0.24 cal.
Gabrielle's Angel Foundation $75,000
Christa Justus/Grant Administrator
14 Penn Plaza, Suite 1704
New York, NY 10122
Leukemia cells reside in the bone marrow in a specialized niche, receiving support from contact with a variety of cells. We propose a novel approach to the identification of genes that are essential for the survival of leukemia cells within their microenvironment and that could be targeted by novel therapies.
THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

Genetic predictors of clinical outcome in MDS

MDS Agreement (Ebert) 06/01/12-5/31/14 0.12 cal.
MDS Foundation $34,000
Susan Hogan
MDS Foundation, Inc.
4573 South Broad Street
Suite 150
Yardville, NJ 08620

The current study will integrate molecular data from multiple MDS centers to develop a new version of the IPSS score that employs both clinical and molecular features.

THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

Targeting CD97 for the treatment of myeloid malignancies

Cancer Research Grant (Ebert) 08/01/2012-7/31/2014 0.60 cal
Claudia Adams Barr Program in Cancer Research $150,000
Candice J. Hachey/Grant Administrator
44 Biney Street, Mayer 1B, Room 25
Boston, MA 02115

The specific aims of this project are designed to validate the relevance of CD97 expression and function in human myeloid malignancies, to examine the biology of CD97 in normal and leukemia cells using definitive murine models, and to develop and test a novel therapeutic antibody targeting CD97.

THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

New Active Projects since award activation

Novel Therapeutic Strategies in Human Myeloid Leukemias—Project 2 and Core A

NIH/NCI (Ebert) 09/16/14-08/31/19 1.82 cal
P01 CA066996 -17 $222,768

The goal of this project is to develop novel approaches to the treatment of acute myeloid leukemia based on combinations of lenalidomide with additional therapies.

Role: PD/PI (Project 2 and Core A)

THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

DFCI/NIH (Subaward) 12/1/14-11/30/15 0.92 cal
CCSG $24,503

This subaward funds a portion of Dr. Ebert's salary to support his administrative role as leader of the leukemia program for the Dana-Farber/Harvard Cancer Center.

Role: Leukemia Group Leader

THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

Understanding and Targeting Spliceosomal-Mutation Hematopoietic Malignancies

The Broad Institute (Abdel-Wahab/Krainer/Ebert) 01/01/15-12/31/16 0.12 cal
STARR CANCER CONSORTIUM $138,333

The aims of this proposal are to understand the biology of *SF3B1* mutations in myelodysplsatic syndrome.

THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

New Pending Projects since award activation

SPORE in Myeloid Malignancies

NIH/NCI(Ebert/Stone) 7/1/16-6/30/21 2.40 cal
1 P50 CA206963-01 $1,957,978(overall project); $206,849 Project 3 and Core A
Myeloid malignancies are cancers which represent overgrowth of bone marrow stem cells that lead to abnormal production of blood cells. Understanding the mechanisms that allow such overgrowth will provide an avenue to develop therapeutic strategies which involve specifically inhibiting these pathways and/or aid the patients own immune system to recognize these abnormal cells. Research in the SPORE will develop new therapies which could be more specifically effective than cytotoxic approaches.
THERE IS NO OVERLAP WITH THE CURRENT APPLICATION.

STEENSMA, DAVID – OTHER SUPPORT

Ended Projects since award activation

None.

New Active Project since award activation

#Aplastic Anemia & MDS International Foundation (Steensma, D.) 07/01/2014 – 06/30/2016 0.00 cal
Supplemental Funding $136,364
The specific aims of the supplemental project are to update the institutional database for MDS patients and merge data with other institutions in the MDS Consortium, and to prospectively capture patient data for the new Precursor Clinic. Role: Principal Investigator

POC: Kathleen Weis, Chief Executive Officer, Aplastic Anemia & MDS International Foundation. 100 Park Avenue Suite 108, Rockville, MD 20850. Phone: (301) 279-7202 ext. 100. Email: weis@aamds.org

#Aplastic Anemia & MDS International Foundation (Steensma, D.) 06/29/2015 – 06/30/2016 0.36 cal
Molecular Correlates of Agent Orange Exposure in United States Military Veterans with Myelodysplastic Syndromes $249,090
The goal of this project is to study individuals exposed to Agent Orange, its contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin, and associated solvents during the Vietnam War era as well as an unexposed control population, in order to better understand the potential contribution of Agent Orange to acquisition of somatic mutations in hematopoietic stem cells and subsequent development of clonal hematopoiesis and myeloid neoplasms. Role: Principal Investigator

POC: Kathleen Weis, Chief Executive Officer, Aplastic Anemia & MDS International Foundation. 100 Park Avenue Suite 108, Rockville, MD 20850. Phone: (301) 279-7202 ext. 100. Email: weis@aamds.org

New Pending Projects Since Award Activation

P50 SPORE (Ebert/Stone) 07/01/2016-06/30/2021
1.20 cal
NIH (BWH) $37,342
SPORE in Myeloid Malignancies – Project 3
The goals of this project are to develop the SF3B1 inhibitor clinical protocol, assist in Dana-Farber/Harvard Cancer Center Institutional Review Board submission and other regulatory matters, enroll and treat patients with myelodysplastic syndromes (MDS) and other myeloid neoplasms on the H3B 8800 protocol, and

collaborate with enrolling co-investigators at other centers. In addition, he will collaborate with the Ebert laboratory in pre-clinical studies, including identifying and providing primary patient samples for in vitro analyses. Role: Site Principal Investigator

R21 (Chai, L.) 09/01/2015 – 08/31/2017 0.60
cal
National Cancer Institute $12,281
A Biomarker to Predict MDS Patient Prognosis
The major goal of this project is to identify and provide patient samples for this study and share expert opinion on MDS patient management. Role: Site Principal Investigator

What other organizations were involved as partners?

Provide the following information for each partnership:

Organization Name: **Dana Farber Cancer Institute, Boston MA**

Partner's contribution to the project: Collaboration with David Steensma, MD

8. SPECIAL REPORTING REQUIREMENTS: None

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Regular Article

MYELOID NEOPLASIA

TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients

Rafael Bejar,[1] Allegra Lord,[2] Kristen Stevenson,[3] Michal Bar-Natan,[4] Albert Pérez-Ladaga,[1] Jacques Zaneveld,[5] Hui Wang,[5] Bennett Caughey,[1] Petar Stojanov,[6] Gad Getz,[6] Guillermo Garcia-Manero,[7] Hagop Kantarjian,[7] Rui Chen,[5] Richard M. Stone,[4] Donna Neuberg,[3] David P. Steensma,[4] and Benjamin L. Ebert[2,6]

[1]Division of Hematology and Oncology, University of California San Diego Moores Cancer Center, La Jolla, CA; [2]Division of Hematology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA; [3]Department of Biostatistics and Computational Biology and [4]Department of Medical Oncology, Division of Hematological Malignancies, Dana-Farber Cancer Institute, Boston, MA; [5]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX; [6]Broad Institute of Harvard and Massachusetts Institute of Technology, Cambridge, MA; and [7]Department of Leukemia, University of Texas MD Anderson Cancer Center, Houston, TX

Key Points

- Higher abundance *TET2* mutations are associated with increased response to hypomethylating agents, particularly when *ASXL1* is not mutated.
- *TP53* and *PTPN11* mutations are associated with shorter overall survival after hypomethylating agent treatment, but do not predict response.

Only a minority of myelodysplastic syndrome (MDS) patients respond to hypomethylating agents (HMAs), but strong predictors of response are unknown. We sequenced 40 recurrently mutated myeloid malignancy genes in tumor DNA from 213 MDS patients collected before treatment with azacitidine (AZA) or decitabine (DEC). Mutations were examined for association with response and overall survival. The overall response rate of 47% was not different between agents. Clonal *TET2* mutations predicted response (odds ratio [OR] 1.99, $P = .036$) when subclones unlikely to be detected by Sanger sequencing (allele fraction <10%) were treated as wild-type (WT). Response rates were highest in the subset of *TET2* mutant patients without clonal *ASXL1* mutations (OR 3.65, $P = .009$). Mutations of *TP53* (hazard ratio [HR] 2.01, $P = .002$) and *PTPN11* (HR 3.26, $P = .006$) were associated with shorter overall survival but not drug response. Murine-competitive bone marrow transplantation followed by treatment with AZA demonstrated that *Tet2*-null cells have an engraftment advantage over *Tet2*-WT cells. AZA significantly decreased this advantage for *Tet2*-null cells ($P = .002$) but not *Tet2*-WT cells ($P = .212$). Overall, *Tet2* loss appears to sensitize cells to treatment with AZA in vivo, and *TET2* mutations can identify patients more likely to respond to HMAs. (*Blood*. 2014;124(17):2705-2712)

Introduction

DNA hypomethylating agents (HMAs) are the only class of drugs approved for the treatment of patients with higher-risk myelodysplastic syndromes (MDS). Azacitidine (AZA) was approved by the Food and Drug Administration (FDA) for MDS in 2004 and was later shown to confer an overall survival benefit compared with supportive care in a randomized phase 3 study.[1] Decitabine (DEC), the deoxynucleotide analog of AZA, was approved for the treatment of MDS in 2006 based on its ability to improve blood counts and decrease bone marrow blasts proportions.[2] However, only 40% to 50% of patients treated with either AZA or DEC experience hematologic improvement (HI) with these agents, and complete responses (CRs) occur in as few as 10% to 15% of treated patients.[3,4] Effective methods for identifying patients who are most likely to respond to treatment with an HMA would be of immediate clinical utility. Clinical features and patient characteristics may help stratify patients according to their response rates, but these models are not sufficiently conclusive to deny eligible patients a trial of AZA or DEC based on their predictions alone.[5,6] Better biomarkers of response to HMAs are needed.

Since the FDA approval of AZA and DEC, our understanding of the molecular genetic basis for MDS has expanded dramatically. Recurrent somatic mutations have been identified in more than 40 genes, and many of these mutated genes have been associated with important clinical measures including overall survival.[7-9] Because mutated genes underlie the pathogenic mechanisms driving the initiation and progression of MDS, they may represent molecular biomarkers of drug sensitivity or resistance. This is exemplified by the observation that MDS with deletions of the long arm of chromosome 5 (del[5q]) have a striking sensitivity to lenalidomide, whereas MDS patients without this lesion are less likely to have a hematologic response and are much less likely to have a cytogenetic or prolonged response.[10] No such cytogenetic correlate has been found for the HMAs, but single-gene mutations involving the pathways targeted by these drugs may be better candidates.

DEC and AZA (which is metabolized into DEC intracellularly) inhibit DNA methyltransferases and decrease the methylation of cytosine residues. Several of the most frequently mutated genes in

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MDS encode proteins involved in the epigenetic regulation of gene expression such as TET2, DNMT3A, and ASXL1. DNMT3A is a de novo DNA methyltransferase and is a potential target of the HMAs. Somatic mutations of DNMT3A have been shown to decrease its activity, suggesting that pharmacologic targets other than DNMT3A are likely mediators of response to AZA and DEC.[11] Loss of function mutations in *TET2* impair the ability of this enzyme to oxidize methylcytosine residues and are associated with altered DNA methylation patterns and decreased 5-hydroxymethylcytosine levels in MDS patient samples.[12,13] A small study of AZA-treated MDS patients using Sanger sequencing to determine the mutation status of *TET2* found that mutations of this gene were associated with a slightly higher rate of response than in wild-type (WT) *TET2* patients.[14] However, the investigators did not examine these samples for additional mutations that might have modified this result and did not use techniques sensitive enough to identify mutations in small-disease subclones. Subclonal mutations in genes associated with an adverse prognosis, including *ASXL1, RUNX1,* and *NRAS*, have been shown to have clinical relevance regardless of their abundance within the dysplastic clone.[8,15] These adverse mutations are often associated with disease progression and may mitigate the value of a sensitizing abnormality if they confer resistance to treatment.

We hypothesize that mutations of individual genes may serve as biomarkers of response for MDS patients treated with HMAs. We used massively parallel sequencing to examine 40 recurrently mutated genes in disease samples from 213 MDS patients treated with AZA or DEC. We examined the association of mutational patterns at different mutant allele fractions with response to treatment and overall survival. We used a competitive murine bone marrow transplant model to test the sensitivity of *Tet2*-null and *Tet2* wild-type (*Tet2*-WT) hematopoietic cells to treatment with AZA.

Materials and methods

Patient samples and response assessment

A total of 213 MDS patients treated with AZA or DEC were included in this study. Samples were obtained from patients treated at the Dana-Farber Cancer Institute (2003-2010, N = 42), the MD Anderson Cancer Center (2003-2010, N = 104), and as part of the DACO-020 (ADOPT) clinical trial of DEC (2005-2006, N = 67). All samples were collected with patient consent under institutional review board–approved protocols in accordance with the Declaration of Helsinki. Response to treatment was assessed using International Working Group (IWG) response criteria revised in 2006. Patients with either a CR, partial response (PR), or HI were considered as "responders" (R, n = 100, 47%), whereas patients described as having "no response," "stable disease," "progressive disease," "death" before response assessment, or "not evaluable" were considered "nonresponders" (NR, n = 113, 53%).

Sample processing, DNA sequencing, and mutation analysis

DNA was extracted from bone marrow mononuclear cells or peripheral blood samples collected before treatment (median 18 days, range 9-119). Whole-genome amplification of DNA for each sample was performed using the REPLI-g kit from QIAGEN. A genotype fingerprint of 22 common single-nucleotide polymorphisms (SNPs) for each sample was generated by MALDI-TOF genotyping (Sequenom). Target regions of 40 genes (supplemental Table 1, available on the *Blood* Web site) and genotype fingerprint regions were enriched using the Agilent SureSelect hybrid capture system according to the manufacturer's instructions. Barcoded samples were pooled in equimolar amounts and subjected to 100 nucleotide paired-end sequencing on an Illumina Hi Sequation 2000. Sequence reads were aligned to the human genome (Build 37) using the Burroughs-Wheeler algorithm.[16] The Genome Analysis Toolkit was used to clean and locally realign reads before calling missense and insertion/deletion variants using MuTect.[17,18] Sample identity was confirmed by matching fingerprint genotype calls. Synonymous variants, noncoding variants more than 6 bases from splice junctions, or variants present in databases of "normal" genomes (dbSNP 132 or NHLBI Exome Sequencing Project) at a population frequency of 1% or more were discarded. Remaining variants were considered candidate somatic mutations.

Competitive murine bone marrow transplants

Age-matched $Tet2^{-/-}$;Mx-Cre^{+} and $Tet2^{+/+}$;Mx-Cre^{+} donor animals (CD45.2) were treated with pIpC (15 μg/g intraperitoneally [IP]) for 3 nonconsecutive days to induce excision of exon 3 of Tet2.[19] Donor bone marrow was harvested 2 weeks post-pIpC and mixed in a 1:2 ratio with bone marrow harvested from 45.1 WT donors (B6.SJL-Ptprca Pepcb/BoyJ; Jackson Labs), and was then transplanted into 45.1 recipients for a total of 1 million cells per recipient. Peripheral blood engraftment was assessed by fluorescence-activated cell sorting at 2 weeks posttransplant, at which point recipient mice were divided into treatment groups (n = 7 per group) and treated with either 5-AZA (2.5 mg/kg IP; Santa Cruz Biotechnology) or vehicle control on the following schedule: 2 weeks on, 2 weeks off. Peripheral blood chimerism and complete blood count were assessed after each round of treatment.

Statistical methods

Categorical variables were compared using the Fisher exact test or χ^2 test as appropriate, whereas continuous variables were compared using the Wilcoxon rank-sum test. A Cochran-Mantel-Haenszel test was used to test for differences in response rate by mutational status controlling for treatment. Unadjusted and adjusted logistic regression models were used to predict response to therapy. Models were adjusted for covariates including age (≥70 y vs <70 y), sex, International Prognostic Scoring System (IPSS) risk group (low/intermediate 1 vs intermediate 2/high) and treatment (AZA vs DEC alone vs DEC ± other). The odds ratio (OR) and 95% confidence intervals (CI) were estimated for the risk group (mutated) and compared with the reference group (WT). The Hosmer and Lemeshow goodness-of-fit test was used to assess model fit of logistic regression models. Overall survival was calculated from the time of treatment to the time of death from any cause, or was censored at the date last known alive and was compared using a log-rank test. Unadjusted and adjusted univariate Cox models were also constructed using the same covariates. For the competitive murine experiments, the percent 45.2 chimerism was calculated for each time point. Error bars indicate standard error of the mean (SEM) for each group, and *P* values for each time point were calculated using a 2-sample Student *t* test. All *P* values reported are 2-sided and considered significant at .05. No adjustments were made for multiple hypothesis testing.

Results

Spectrum of mutations

We examined tumor samples collected from 213 patients from 3 different sites before treatment with AZA, DEC, or DEC + another agent. There were no significant differences in pretreatment patient characteristics by treatment site (Table 1) or baseline characteristics as shown in Table 2. Frequently mutated regions of 40 genes previously shown to be somatically mutated in patients with MDS were subject to hybrid capture and massively parallel sequencing (supplemental Table 1). These include the most frequently mutated splicing factors, kinase signaling genes, transcription factors, and epigenetic regulators such as *TET2*, *DNMT3A*, *ASXL1*, and *EZH2*. With this panel, we identified one or more mutations in 39 genes

Table 1. Patient characteristics and treatments received by *TET2* mutational status

	N (%)	*TET*-WT	*TET2*-mut	*P* value*
N	213	155	58	
Treatments received				
AZA alone	42 (20)	30 (19)	12 (21)	.60
DEC alone	144 (68)	103 (66)	41 (71)	
DEC + other	27 (13)	22 (14)	5 (9)	
Age, ≥70 y	103 (48)	72 (46)	31 (53)	.44
Sex				
Male	155 (73)	118 (76)	37 (64)	.085
Female	58 (27)	37 (24)	21 (36)	
FAB				
RA	30 (14)	19 (12)	11 (19)	.13
RARS	24 (11)	15 (10)	9 (16)	
RAEB	125 (59)	97 (63)	28 (48)	
RAEB-t	6 (3)	4 (3)	2 (3)	
CMML	21 (10)	13 (8)	8 (14)	
Other	7 (3)	7 (5)	0 (0)	
IPSS risk group				
Low	11 (5)	5 (3)	6 (10)	.019
Int-1	86 (40)	56 (36)	30 (52)	
Int-2	76 (36)	61 (39)	15 (26)	
High	37 (17)	31 (20)	6 (10)	
Unknown	3 (1)	2 (1)	1 (2)	
Cytogenetics				
Normal or –Y alone	107 (50)	68 (44)	39 (67)	.022
Complex	51 (24)	45 (29)	6 (10)	
−7/7q- isolated or +1	14 (7)	12 (8)	2 (3)	
+8 isolated	11 (5)	8 (5)	3 (5)	
20q- isolated	7 (3)	6 (4)	1 (2)	
5q- isolated or +1	3 (1)	3 (2)	0 (0)	
Other	13 (6)	9 (6)	4 (7)	
Unknown	7 (3)	4 (3)	3 (5)	

*Test includes only known categories, χ^2 test used for cytogenetics.

(Figure 1). In total, 94% of patients had a mutation in at least one recurrently mutated gene. The most frequently mutated genes were *ASXL1* (46%), *TET2* (27%), *RUNX1* (20%), *TP53* (18%), and *DNMT3A* (16%) followed by the splicing factor genes *SRSF2* (16%), *SF3B1* (15%), and *U2AF1* (14%).

The frequency of mutations identified in these genes was largely similar to those identified in other MDS patient cohorts. Only *ASXL1* mutations were more frequent compared with prior studies, many of which examined a greater proportion of lower-risk patients without transfusion dependence; used less sensitive Sanger sequencing of *ASXL1*; excluded unannotated missense mutations; or excluded insertions in a homopolymeric tract near amino acid 642.[7-9] Other previously observed patterns of mutations were identified in this cohort including the paucity of *ASXL1* mutations in *SF3B1* mutant samples, the near mutual exclusivity of splicing factor mutations, and the lower rate of other gene mutations in patients with *TP53* mutations (supplemental Figure 1).[20,21] As expected, mutations of *TET2*, *ASXL1*, *NRAS*, *EZH2*, and *SRSF2* were overrepresented in chronic myelomonocytic leukemia (CMML) cases, *SF3B1* mutations were predominantly in refractory anemia with ring sideroblasts (RARS) cases, and mutations of *TP53*, *IDH1*, and *IDH2* were relatively underrepresented in refractory anemia (RA)/RARS patients (supplemental Figure 1). The variant allele fractions (VAFs) of mutations were not uniform and varied greatly for individual genes (Figure 2). For example, splicing factor abnormalities had higher median variant allele fractions, whereas the VAFs for mutations in tyrosine kinase signaling genes were lower, indicative of their frequent presence in disease subclones. Mutations of all genes included some variants present only at low abundance.

Clinical findings, variant allele fraction, and response rates

The overall response rate of patients in the study was 47%, with 31% achieving CR according to IWG criteria revised in 2006 (Table 2). There was no significant difference in response by treatment regimen ($P = .96$) or source of sample ($P = .36$). IPSS risk groups and cytogenetic abnormalities were not associated with response rate. The only clinical feature significantly associated with response rate was FAB classification ($P = .008$), driven largely by the high response rate of CMML patients (17/21, 81%). Thirty-five percent of RA/RARS patients achieved a response compared with 47% of refractory anemia with excess blasts (RAEB) patients. No differences were detected in the time to response for each mutation.

In a prior study by Itzykson et al, mutations of *TET2* detected by Sanger sequencing were found to predict a nearly twofold greater response rate with AZA.[14] In our cohort, *TET2* mutant patients showed only a trend toward increased response rates compared with WT (55% vs 44%; OR 1.58 [0.86-2.89], $P = .14$) and no other mutated gene was associated with a significantly improved overall response rate in univariate analyses (Table 3, supplemental Table 2, and supplemental Figure 2). However, the VAF for mutations of *TET2* and several other genes spanned a wide range of values including many likely to be below the detection limit for Sanger sequencing (Figure 2). We hypothesized that mutations capable of sensitizing cells to HMAs are more likely to be associated with a clinical response to treatment when they are present in a major disease clone. For example, even complete elimination of a clone representing <20% of bone marrow cells might not have any effect on the assessment of clinical response. Therefore, we repeated our analysis with mutations present at a VAF of <10% treated as if they

Table 2. Response vs patient characteristics and treatment

	Total	Nonresponders, n (%)	Responders, n (%)	*P* value*
N	213	113 (53)	100 (47)	
Treatment				
AZA alone	42 (20)	22 (52)	20 (48)	.96
DEC alone	144 (68)	76 (53)	68 (47)	
DEC + Other	27 (13)	15 (56)	12 (44)	
Sex				
Male	155 (73)	82 (53)	73 (47)	.99
Female	58 (27)	31 (53)	27 (47)	
Age				
<70 y	110 (52)	64 (58)	46 (42)	.13
≥70 y	103 (48)	49 (48)	54 (52)	
FAB				
RA	30 (14)	20 (67)	10 (33)	.008*
RARS	24 (11)	15 (63)	9 (38)	
RAEB	125 (59)	65 (52)	60 (48)	
RAEB-t	6 (3)	4 (67)	2 (33)	
CMML	21 (10)	4 (19)	17 (81)	
Other	7 (3)	5 (71)	2 (29)	
IPSS risk group				
Low/Int-1	97 (46)	53 (55)	44 (45)	.78†
Int-2/High	113 (53)	59 (52)	54 (48)	
Unknown	3 (1)	1 (33)	2 (67)	
Cytogenetics				
Normal	104 (49)	49 (47)	55 (53)	.31
Complex	51 (24)	28 (55)	23 (45)	
Other	51 (24)	31 (61)	20 (39)	
Unknown	7 (3)	5 (71)	2 (29)	

*Test includes only known categories.
†No difference was observed between the 4 individual IPSS categories ($P = .24$).

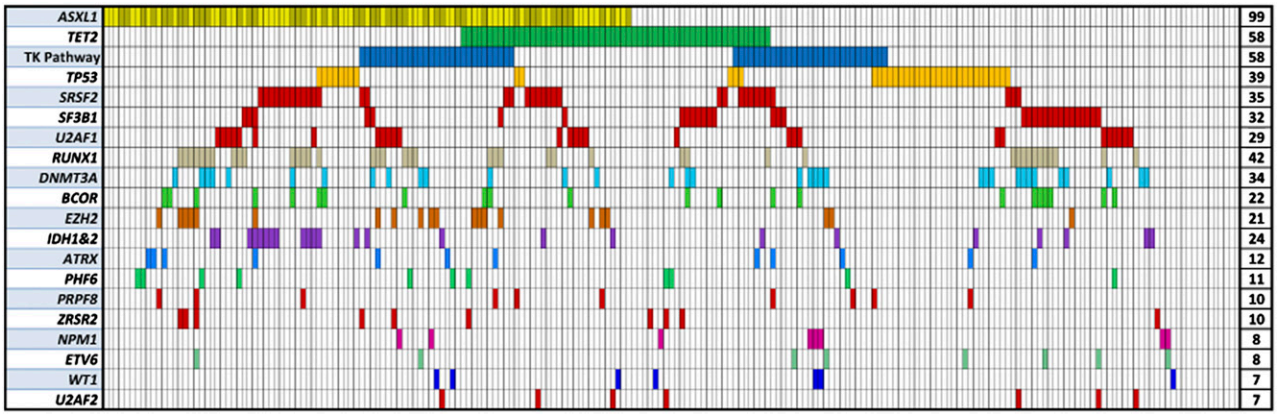

Figure 1. Spectrum of mutations in 213 patients in select MDS-associated genes. Each column represents an individual patient sample, and each colored cell represents a mutation of the gene or gene group listed to left of that row. The number of mutations for each row is indicated in the column to the right. Darker bars in the *ASXL1* row indicate patients with a p.G642fs mutation. TK Pathway = *NRAS*, *KRAS*, *CBL*, *CBLB*, *JAK2*, *PTPN11*, *BRAF*, *MPL*, and *KIT*.

were unmutated. For heterozygous mutations, this cutoff represents variants present in <20% of the sample cellularity and is at the estimated limit of sensitivity for Sanger sequencing. In this revised analysis considering VAF, mutations of *TET2* were associated with a significantly higher response rate compared with WT (60% vs 43%; OR 1.99 [1.05-3.80], $P = .036$; adjusted OR 1.98 [1.02-3.85], $P = .044$), and comparable with that shown by Itzykson et al (Table 3, supplemental Table 2, and supplemental Figure 2).

When all VAFs were considered, only mutations of *CBL*, which were often of low VAF, were associated with a lower rate of response compared with WT (14% vs 49%) in this analysis (OR 0.17 [0.04-0.79], $P = .023$; adjusted OR 0.18 [0.04-0.82], $P = .027$) but was not significant when low VAF mutations were considered WT (20% vs 48%) (OR 0.27 [0.06-1.30], $P = .10$).

By sequencing multiple genes, we had the opportunity to determine whether mutations in additional genes could modulate the response rates of *TET2*-mutant patients. We focused on the subset of patients defined by their *TET2* and *ASXL1* mutation status (at any VAF) because these contained enough patients for a meaningful statistical analysis. Patients with mutated *TET2* and unmutated *ASXL1* demonstrated an increased overall response rate compared with all others (65% vs 44%; OR 2.37, [1.00-5.58], $P = .049$) (Table 3). This effect was more pronounced when mutations were required to have a VAF ≥10% (74% vs 44%; OR 3.65, [1.38-9.67], $P = .009$), representing >10% of patients in this cohort (Table 3).

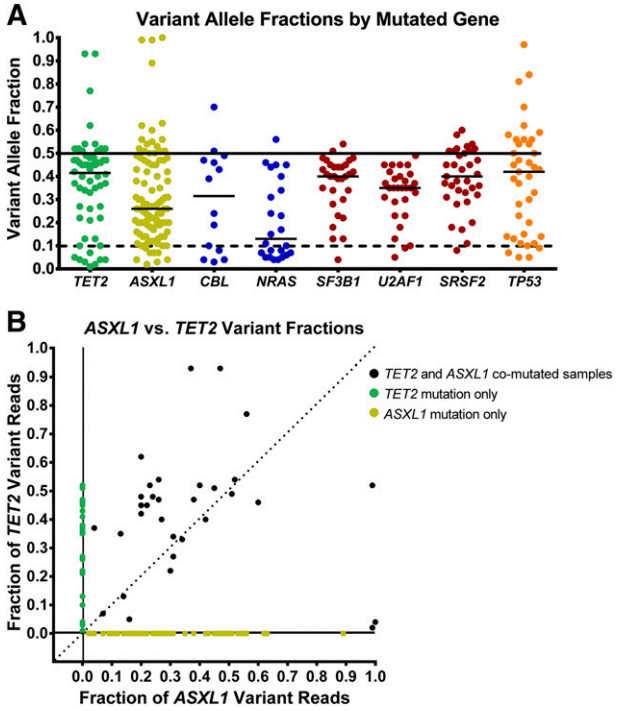

Figure 2. Variant allele frequencies in selected genes. (A) Quantitative measure of variant-containing reads estimates the abundance of these mutations (uncorrected for allele copy number). Mutations of *TET2* (green), *TP53* (orange), and splicing factor genes (red) are often present in the dominant clone, whereas mutations of tyrosine kinase–signaling genes (blue) are often present in smaller clones. Mutations of *ASXL1* (yellow) are more widely distributed. (B) Analysis of samples with both *TET2* and *ASXL1* mutations indicate that *ASXL1* mutations are most often codominant with, or smaller than, *TET2*-mutant clones.

In vivo model of AZA response in Tet2$^{-/-}$ cells

The observed association between *TET2* mutations and response to treatment could be mediated directly by *TET2* loss-of-function or by indirect or cell-extrinsic effects. To test whether *TET2* loss-of-function can sensitize cells to HMAs, we performed a competitive murine bone marrow transplant experiment using hematopoietic cells from *Tet2*-null and WT littermate donors. As expected, equal numbers of CD45.2^{+} cells transplanted into CD45.1^{+} recipients resulted in greater engraftment of *Tet2*-null cells by 2.5 weeks posttransplant, before treatment with AZA. There was no difference in peripheral blood counts between groups at this time point. Treatment with AZA (2.5 mg/kg M-F × 2 weeks) or vehicle was begun on day 20 posttransplant and repeated starting on days 48, 76, and 104. Regardless of genotype, AZA-treated animals exhibited significant decreases in white blood cell and hematocrit levels and an initial drop in peripheral blood chimerism. For several subsequent cycles, AZA-treated *Tet2*-null cells maintained a significantly decreased representation in peripheral blood, whereas *Tet2*-WT cells did not (Figure 3).

Associations with overall survival

Traditional prognostic models like the IPSS and Revised IPSS (IPSS-R) are based on patient cohorts examined only until they receive disease-modifying therapies such as HMAs or they undergo stem cell transplantation. Response to specific treatments could significantly alter the prognostic impact of adverse disease

Table 3. Association of gene mutations with response rate in logistic regression analysis

Mutated gene*	Unadjusted OR (95% CI)	*P* value	Adjusted† OR (95% CI)	*P* value
Mutations with VAF ≥10%				
TET2-mut vs *TET2*-WT	1.99 (1.05, 3.80)	.036	1.98 (1.02, 3.85)	.044
ASXL-mut vs *ASXL1*-WT	0.69 (0.40, 1.20)	.19	0.68 (0.38, 1.19)	.17
TET2-mut + *ASXL1*-WT vs other	3.65 (1.38, 9.67)	.009	3.64 (1.35, 9.79)	.011
TET2-mut + *ASXL1*-WT vs both WT	3.40 (1.24, 9.35)	.011	3.36 (1.20, 9.38)	.013
TET2-WT + *ASXL1*-mut vs both WT	0.77 (0.41, 1.46)	.35	0.80 (0.39, 1.46)	.39
TET2-mut + *ASXL1*-mut vs both WT	1.11 (0.48, 2.61)	.62	1.07 (0.44, 2.61)	.59
CBL-mut vs *CBL*-WT	0.27 (0.06, 1.29)	.10	0.28 (0.06, 1.40)	.12
Including all mutations				
TET- mut vs *TET2*-WT	1.58 (0.86, 2.89)	.14	1.60 (0.85, 3.02)	.15
ASXL1-mut vs *ASXL1*-WT	0.77 (0.45, 1.32)	.34	0.74 (0.42, 1.30)	.29
TET2-mut + *ASXL1*-WT vs other	2.37 (1.00, 5.58)	.049	2.40 (0.99, 5.79)	.051
TET2-mut + *ASXL1*-WT vs both WT	2.27 (0.91, 5.63)	.055	2.27 (0.89, 5.79)	.056
TET2-WT + *ASXL1*-mut vs both WT	0.86 (0.45, 1.64)	.16	0.84 (0.43, 1.62)	.15
TET2-mut + *ASXL1*-mut vs both WT	1.06 (0.47, 2.38)	.67	1.04 (0.45, 2.44)	.68
CBL-mut vs *CBL*-WT	0.17 (0.04, 0.79)	.023	0.18 (0.04, 0.82)	.027

*Reference group is listed second.

†Adjusted for sex, age (<70, ≥70 y), IPSS (Low/Int1 vs Int2/High), and treatment (AZA alone vs DEC alone vs DEC + other); none of the Hosmer and Lemeshow tests indicated a lack of fit for each model.

features or genetic alterations. We explored the relationship between mutation status and overall survival in the subset of patients with available survival data. Of these 146 patients (69%) in our cohort, 119 died during follow-up. The median follow-up for patients remaining alive was 3.8 years (95% CI, 3.1-5.8). Despite its association with response, *TET2* mutation status was not associated with overall survival ($P = .56$), consistent with the finding in Itzykson et al (Figure 4A). Mutations of *TP53* were associated with lower overall survival (21% of patients; HR 2.01 [1.29-3.14], $P = .002$; adjusted HR 1.91 [1.20-3.05], $P = .007$; Figure 4B) as were the much rarer mutations of *PTPN11* (4% of patients, HR 3.26 [1.41-7.58], $P = .006$; adjusted HR 2.47 [0.98-6.26], $P = .056$; Figure 4C).

Overall survival in patients with complex karyotypes was strongly associated with *TP53* mutation status (Figure 4D). Patients with both a complex karyotype and a *TP53* mutation had a median survival of only 0.9 years. In contrast, patients with complex cytogenetics and no *TP53* mutation had an overall survival of 1.3 years, which was not different from patients with karyotypes other than complex (median 1.8 years, $P = .28$). This indicates that the adverse prognostic value ascribed to the complex karyotype is largely driven by its frequent association with *TP53* mutations, which could be used to better refine disease risk in this patient population.

Discussion

In our study, the presence of *TET2* mutation at >10% allele burden predicted an increased response to HMAs, particularly in the subset that lacked similarly abundant mutations of *ASXL1*. To achieve this result, we examined tumor samples from 213 MDS patients collected before treatment with HMAs for mutations in 40 genes known to be recurrently mutated in MDS. The patients in our cohort were representative of those studied in clinical trials of AZA and DEC in terms of predicted disease risk and severity of cytopenias. Overall response rates were just under 50% and did not differ by the type of drug patients received. Using sensitive quantitative sequencing

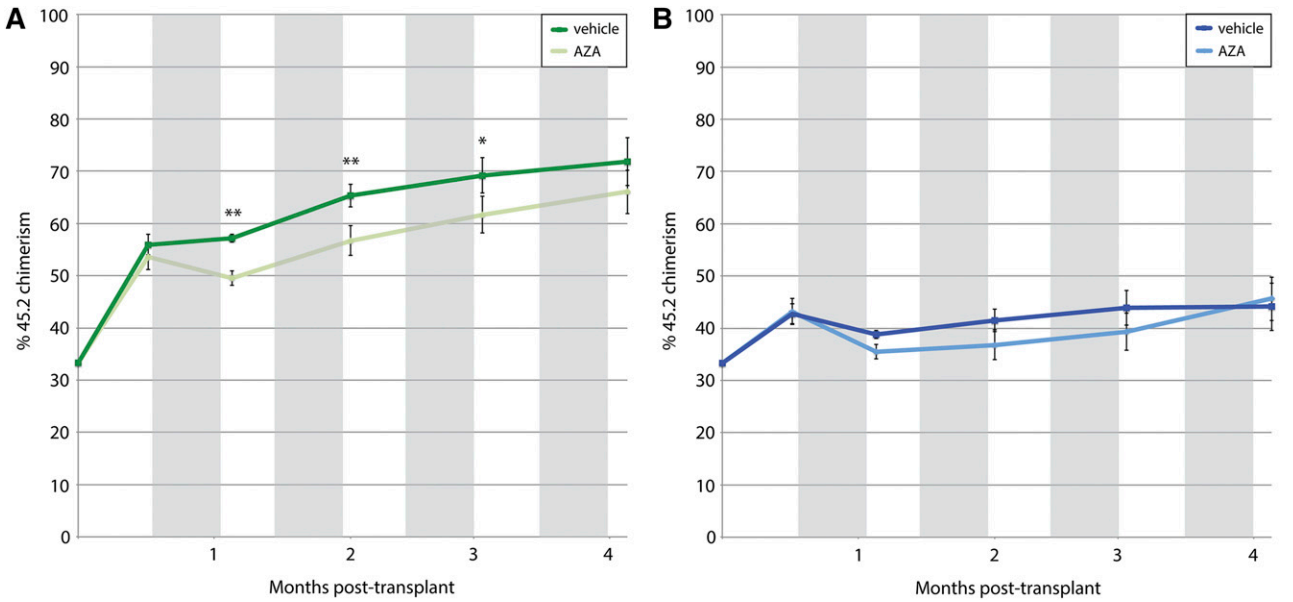

Figure 3. Peripheral blood chimerism. Shown over time after competitive bone marrow transplantation with cells from 45.2 *Tet2*-null mice (A) and 45.2 *Tet2*-WT mice (B). Gray bars indicate periods of treatment with AZA or vehicle. *Tet2*-null cells show increased chimerism compared with *Tet2*-WT cells. Treatment with AZA significantly decreases chimerism in the *Tet2*-null recipient mice only. *$P < .05$, **$P < .01$.

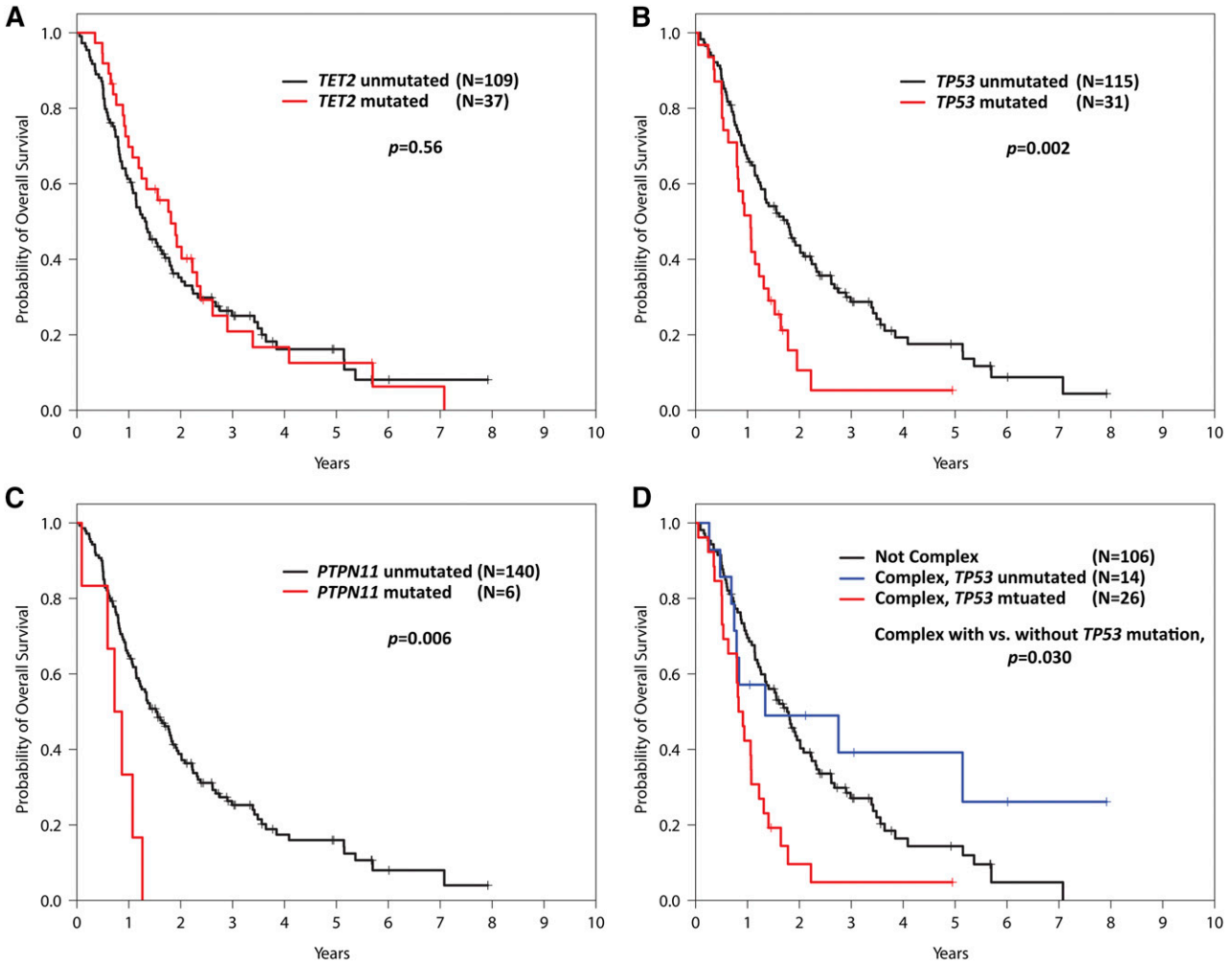

Figure 4. Kaplan-Meier curves for overall survival in the 146 out of 213 study patients with survival data. (A) Survival of patients with and without *TET2* mutations. (B) Survival of patients with and without *TP53* mutations. (C) Survival of patients with and without *PTPN11* mutations. (D) Survival of complex karyotype patients with and without *TP53* mutations vs patients without complex karyotypes.

techniques, we were able to identify mutations in >90% of patients in patterns similar to those seen in prior multigene studies of MDS.

Our findings are consistent with those of Itzykson et al, who previously reported that 11 of 13 (85%) MDS patients with *TET2* mutations detected by Sanger sequencing responded to treatment with AZA compared with a 52% response rate for their overall cohort of 86 patients. In that study, mutations in other genes were not examined, and small subclonal *TET2* mutations likely went undetected. Our broader and more sensitive multigene analysis similarly identified *TET2* mutations as predictive of response to HMAs in a larger cohort of patients. Surprisingly, consideration of mutations in other genes did not reveal additional predictors of favorable response, and inclusion of low VAF mutations weakened the association between *TET2* mutation status and response rate. However, our approach identified the 10% of patients with mutated *TET2* and WT *ASXL1* as the group most likely to respond to treatment. Potential explanations for this finding include partial resistance to HMAs caused by *ASXL1* mutations. In this model, the *ASXL1*-mutated subclone would be expected to grow in size during disease progression, or relapse, and might confer primary resistance. *ASXL1*-mutated patients with WT *TET2* did have a lower likelihood of response, but this was not statistically significant (OR 0.63 [0.35-1.15], $P = .13$). Alternatively, we observed that *ASXL1* mutations were often subclonal or at a lower VAF than *TET2* mutations in comutated patients (Figure 2C). The acquisition of secondary mutations (of which *ASXL1* was the most frequent) could indicate more clonally progressive disease that might be inherently more resistant to treatment.

The mechanism by which *TET2* mutations might influence response to HMAs is not clear. Altered methylation has been observed in patients with *TET2* mutations and in animal models of *Tet2* loss. However, measurement of pretreatment DNA methylation by itself has not been found to be predictive of response to HMAs.[22] In our murine bone marrow transplant experiment, exposure to AZA preferentially decreased the clonal advantage associated with loss of *Tet2* function. This effect may be associated with a greater AZA sensitivity in more actively cycling cells because AZA results in cell division–dependent passive demethylation of DNA. Mice with hematopoietic *Tet2* loss are known to have increased myeloid progenitor proliferation.[19,23-25]

An important finding of our study was that no pattern of mutation was strongly associated with a lack of response to treatment. Responses to HMAs were observed even in patients with mutations that confer a very poor prognosis. Therefore, our data indicate that mutation information alone should not be used as a basis for denying therapy with an HMA if treatment is indicated. Studies examining samples collected at multiple time points are needed to identify mutations predictive of acquired resistance or relapsed disease.[26-28]

The association between molecular or clinical biomarkers and HMA response may be confounded by the variations in enzymes responsible for the activation and metabolism of AZA and DEC.[11,29-31] Patients who demonstrate significant hypomethylation of blood-cell DNA after treatment with AZA or DEC (indicating sufficient exposure to target DNA methyltransferases) may be more likely to have a clinically significant response.[22,32] It is possible that the predictive value of cell-intrinsic somatic mutations may be enhanced if controlled for cell-extrinsic variables such as effective dose of HMAs received and activated in cells.

Survival data were collected for more than two-thirds of our cohort. Mutation profiles capable of predicting response to HMAs were not associated with differences in overall survival (Figure 3). However, mutations in 2 genes that were not predictive of response, *TP53* and the rarer *PTPN11*, were each associated with decreased overall survival. The majority of *TP53* mutant patients had a complex karyotype, a known adverse risk factor associated with shorter overall survival. More than half of our complex-karyotype patients harbored a *TP53* mutation (32/51), and these patients had a very short overall survival (median 0.9 years). However, complex karyotype patients without a detectable *TP53* mutation had an overall survival that was no different from the group of patients with noncomplex karyotypes. This indicates that the negative prognostic significance attributed the complex karyotype can be better explained by the *TP53* mutation status of these patients and validates the results of recent studies in MDS and AML.[7,33] In addition to *TP53*, mutations in any of 4 additional genes—*RUNX1*, *ASXL1*, *EZH2*, or *ETV6*—were found to predict shorter overall survival than expected by examining clinical features alone. However, mutations of these 4 genes were not found to be prognostically adverse in this cohort of treated patients (supplemental Figure 3). In contrast, samples for our previous study were collected before the approval of AZA and DEC and therefore came largely from untreated patients. Our results suggest that treatment with HMAs may partially abrogate the adverse prognostic impact of these lesions. If validated, our finding would form a justification for treating patients whose adverse prognosis is driven by mutations in these genes.

The clinical implications of our findings are that response to hypomethylating therapy can be predicted in a subset of patients using molecular genetic features. A more robust predictor might be created by incorporating clinical findings or other biomarkers.[5,34,35] Indeed, the Groupe Français des Myélodysplasies has presented a clinically and cytogenetically based prognostic model for AZA-treated patients, although its predictive power is unclear.[5,6,36] As with these clinical measures, no mutations identified in our study were reliably strong predictors of primary resistance to treatment in a large number of patients. Therefore, there is no genetic rationale for denying MDS patients the opportunity to be treated with AZA or DEC based on our findings, particularly because there are few alternative therapies approved for this patient population.

In conclusion, means of reliably predicting response to HMAs would be of clinical benefit in the care of patients with MDS. Our study demonstrates that mutation profiles can help in this effort to some extent. Studies examining the mechanism by which these biomarkers might mediate sensitivity or resistance to treatment would be of clinical value and could lead to the discovery of additional therapeutic targets in MDS.

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Authorship

Contribution: R.B., B.L.E., and D.P.S. designed the study; M.B.-N., R.M.S., G.G.-M., H.K., and D.P.S. collected samples, curated clinical data, and edited the manuscript; R.B., A.P.-L., J.Z., H.W., B.C., and R.C. carried out DNA sequencing; P.S., G.G., J.Z., and R.B. performed DNA sequencing analysis; A.L. performed the murine experiments; R.B., K.S., and D.N. carried out the statistical analysis; and R.B., B.L.E., K.S., and D.N. wrote the manuscript, which was edited by all authors.

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Correspondence: Benjamin Ebert, Brigham and Women's Hospital, 1 Blackfan Circle–Karp CHRB 5.211, Boston, MA 02115; e-mail: bebert@partners.org; and Rafael Bejar, Moores Cancer Center, University of California, San Diego, 3855 Health Sciences Dr MC 0820, La Jolla, CA 92093-0820; e-mail: rabejar@ucsd.edu.

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TET2 mutations predict response to hypomethylating agents in myelodysplastic syndrome patients

Rafael Bejar, Allegra Lord, Kristen Stevenson, Michal Bar-Natan, Albert Pérez-Ladaga, Jacques Zaneveld, Hui Wang, Bennett Caughey, Petar Stojanov, Gad Getz, Guillermo Garcia-Manero, Hagop Kantarjian, Rui Chen, Richard M. Stone, Donna Neuberg, David P. Steensma and Benjamin L. Ebert

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